

## **TITLE OF THE INVENTION**

METHOD FOR DETERMINING ACTIVITY OF CELL CYCLE  
REGULATORY FACTOR AND METHOD FOR DIAGNOSING  
CANCER USING THE SAME

### **5 CROSS-REFERENCE TO RELATED APPLICATION**

This application is related to Japanese Patent  
Application No. 2001-37115 filed on February 14, 2001, whose  
priority is claimed under 35 USC § 119, the disclosure of  
which is incorporated by reference in its entirety.

### **10 BACKGROUND OF THE INVENTION**

#### **1. Field of the Invention**

The present invention relates to a method for  
determining the activity of a cell cycle regulatory factor without  
using a radioisotope and a method for diagnosing a cancer  
**15** using the method.

#### **2. Description of Related Art**

Cell proliferation is a fundamental and important  
feature of living things. The cell proliferation involves division  
of a single cell into two daughter cells, and somatic cells divide  
**20** through a plurality of sequential reactions including growth of  
cells, replication of DNAs, distribution of chromosomes and  
division of cells. This chain of sequential reactions is referred  
to as a cell cycle. In the case of eucaryotic cells, the cell cycle  
is divided into four phases, that is, a synthetic (S) phase  
**25** during which the replication of DNAs takes place, a mitotic (M)

phase during which the division of cells takes place, a gap 1 (G1) phase which is an interphase from the M phase to the next S phase and a gap 2 (G2) phase which is an interphase from the S phase to the next M phase. In the G1 phase, cells receive a signal for proliferation, prepare for the replication of DNAs, and make metabolism and growth which are necessary for the division of cells. In the G2 phase, the cells prepare for the division. In the G1 phase, a transit point is experimentally assumed which is called an R point (restriction point) for mammalian cells and START for yeast. Typically, cells multiply in response to proliferation signals from the outside. The cells receive the signals in the G1 phase and progress the cell cycle. After passing through a certain point in the G1 phase, the cell cycle progresses from the S phase to the G2 phase, the M phase and then the G1 phase without stopping even if the proliferation signals are not received any longer. This certain point is the R point or START, and is a so-to-speak point at which the entering into cell cycle progression is determined. Further, the cells can leave the cell cycle and enter a resting (G0) phase during which the cell do not grow or multiply. Experimentally, the cells entering the resting phase, if given a suitable signal, can be returned to the G1 phase and induced to grow and divide again. It is considered that a lot of non-growing and non-multiplying cells of multicellular organisms are in the G0 phase.

There mainly exist two groups of cell cycle regulatory factors in cells. One is a group of kinases which are positive regulatory factors and are referred to as cyclin-dependent kinases (CDKs), and the other is a group of CDK inhibitors (CDKIs) which are negative regulatory factors. The CDKs exist in cytoplasm as inactive form. The CDKs are activated, e.g., phosphorylated, and move into nuclei in the cells. In the nuclei, the CDKs bind to cyclin molecules to form complexes with cyclin (referred to as activated CDKs hereinafter) and positively regulate the progress of the cell cycle at various steps of the cell cycle. On the other hand, the CDKIs inactivate the CDKs by bidding to the activated CDKs or CDK simple substances, thereby regulating the cell cycle negatively.

There are now known seven types of CDKs, i.e., CDK1, CDK2, CDK3, CDK4, CDK5, CDK6 and CDK7 to which different cyclins are bound. More particularly, CDK1 binds to cyclin A or B, CDK2 binds to cyclin A or E, and CDK4 and CDK6 bind to cyclin D1, D2 or D3, to be activated. The activated CDKs control specific phases of the cell cycle. The following table 1 shows CDKs concerning the control of the cell cycle, cyclins which functionally bind to the CDKs, and phases of the cell cycle during which the activated CDKs act.

Table 1

CDKs	Cyclins binding to CDKs	Phases of cell cycle in which activated CDKs act
CDK4, CDK6	Cyclin D1, D2, D3	G1
CDK2	Cyclin E	Transitional period from G1 to S
CDK2	Cyclin A	S
CDK1	Cyclin A, B	Transitional period from S to M, M

Thus the cell cycle is controlled and the cell proliferation is regulated by activation of different types of CDKs. The activated CDKs are enzymes which phosphorylate serine residue and threonine residue in a protein as a substrate. In an in-vitro reaction system, the activated CDK1 and CDK2 react well on histone H1 as a substrate and the activated CDK 4 and CDK6 react well on Rb (retinoblastoma protein) as a substrate. In an in-vivo cell cycle regulation, it is considered that the activated CDKs require Rb as a physiologic substrate, but it is not known what other proteins act as substrates.

As described above, the CDKs and cyclins regulate the cell cycle in close association with each other. The multiplication of cyclin D1 gene is observed in a great number of cases of esophageal cancer, while over expression of cyclin D1 gene is observed in a great number of cases of stomach

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cancer and colon cancer. On the other hand, the multiplication of cyclin E gene is observed in stomach cancer and colon cancer but is not observed in esophageal cancer. Excessive expression of cyclin E in stomach and large bowel  
5 takes place with great frequency in cases of adenoma and adenocarcinoma and shows a significant correlation with malignancy such as invasion, progress of stages, metastasis and the like. The expression and kinase activity of CDK1 are remarkably accelerated in most cases of stomach cancer and  
10 colon cancer as compared with normal mucosal tissue. It is known that augmented expression of cyclin genes correlates with the progress and malignancy of various cancers (see Wataru Yasui, Sysmex Journal Web., p.1 to p.10, vol.1, 2000).

Therefore, it is expected that measuring the activity of  
15 the individual species of CDKs will provide good indices of the type and malignancy of diseases related to the control of the cell cycle. In other words, generally at the R point, the expression of CDK2 decreases and the cell cycle arrest and the division of cells is controlled. However, if the expression of  
20 CDK2 increases at the R point, it means that the cell cycle fails to stop, i.e., it means a state of a disease such as cancer. If the expression of CDK4 or CDK6 increases, stomach cancer or colon cancer may be expected because stomach cancer and colon cancer involve accelerated gene expression of the cyclin  
25 D1 which bind specifically to CDK4 or CDK6. Thus, it is

considered to be possible to determine the type of cancer.

Usually, the activity of the CDKs is determined using radioisotopes. More particularly, in the presence of a CDK which is extracted from a cell lysate by an

5 immunoprecipitation method using an anti-CDK antibody and whose activity is unknown,  $^{32}\text{P}$ -labelled adenosine 5'-O-(3-triphosphate) (ATP) is reacted with serine residue or threonine residue in a substrate to introduce monophosphate group derived from the  $^{32}\text{P}$ -labelled ATP. The amount of  $^{32}\text{P}$   
10 taken by the substrate is detected by autoradiography or by a scintillation counter. Thereby the amount of the phosphorylated substrate is measured and the activity of the CDK is calculated from the amount of the phosphorylated substrate.

15 This method requires careful attention in handling the substance and in disposal of waste liquid since it uses  $^{32}\text{P}$  which is a radioisotope.

#### **SUMMARY OF THE INVENTION**

Accordingly, are desired a method for measuring cell  
20 cycle regulatory factors accurately without using radioisotopes and a method for diagnosing cancer on the basis of measurement results.

The present invention is to provide a method for determining the activity of a cell cycle regulatory factor  
25 comprising the steps of:

preparing a sample for measuring a cyclin-dependent kinase/cyclin complex from cells;

reacting adenosine 5'-O-(3-thiotriphosphate) (ATP- $\gamma$  S) with a substrate for the cyclin-dependent kinase in presence of the sample in order to introduce a monothiophosphate group into a serine or threonine residue of the substrate;

labeling the substrate by coupling a labeling fluorophore or a labeling enzyme with a sulfur atom of the introduced monothiophosphate group;

measuring the amount of fluorescence from the labeling fluorophore labeling the substrate, or reacting the labeling enzyme labeling the substrate with a substance which generates an optically detectable product by reaction with the labeling enzyme and optically measuring the amount of the generated product; and

calculating the activity of the cyclin-dependent kinase from the measured amount of fluorescence or the measured amount of the generated product with reference to a pre-produced reference curve.

Further, the present invention is to provide a method of diagnosing a cancer based on a result obtained by determination.

These and other objects of the present application will become more readily apparent from the detailed description given hereinafter. However, it should be understood that the

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detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become  
5   apparent to those skilled in the art from this detailed description.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows a reference curve representing a relation between the amount (ng/slot) of biotinized actin and the  
10   amount of fluorescence (count) obtained in Example 1;

Fig. 2 shows a reference curve representing a relation between the amount (ng/slot) of biotinized actin and the amount of fluorescence (count) obtained in Example 2;

Fig. 3 shows a fluorescent band obtained in Example  
15   3;

Fig. 4 shows a reference curve for measuring the activated CDK2;

Fig. 5 shows a fluorescent band obtained in Example 5; and

20   Fig. 6 is a graphical representation obtained in Example 6.

#### **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

For carrying out the method of the present invention, a sample is first prepared.

25   In the present invention, the sample which contains a



cyclin-dependent kinase (CDK)/cyclin complex may contain a single type or plural types of CDK/cyclin complexes, but may preferably contain a single type of CDK/cyclin complex.

The sample which contains a CDK/cyclin complex (referred to as an activated CDK hereinafter) and is used in the method of the present invention is prepared by solubilizing cells and separating a sample containing the activated CDK to be determined from a liquid containing the solubilized cells.

CDKs usable in the present invention include CDK1, CDK2, CDK3, CDK4, CDK5, CDK6 and CDK7.

#### (1) Step of Solubilizing Cells

The sample is prepared from cells derived from animals including human beings such as a tissue sample (e.g., a biopsy sample, a surgically resected sample, etc.). The sample is to be tested as to whether or not it contains a CDK/cyclin complex as well as its activity. Since simple CDKs exist in cytoplasm and turn into activated CDKs by binding to cyclins in nuclei, cells need to be solubilized to extract the activated CDKs.

Accordingly, the cells are first solubilized by chemically or physically destroying the cell membranes and nuclear envelopes thereof. More particularly, the cells are preferably pulverized using a Waring blender, sucked and discharged using a syringe or ultrasonicated in a buffer containing a surfactant, a protease inhibitor and a

phosphatase inhibitor, for example.

The surfactant is used for destroying cell membranes and nuclear envelopes so that intracellular substances can be taken out. However, the surfactant should have such a surface active property that the activated CDK is not decomposed. Examples thereof include Nonidet P-40, Triton X-100, deoxycholic acid and CHAPS. The concentration of the surfactant is preferably 1 w/v% or less.

The protease inhibitor is used for preventing the CDK and cyclin molecule, which are proteins, from being destroyed when mixed with intracellular substances when the cell membranes and nuclear envelopes are destroyed. Examples thereof include a mixture of a metalloprotease inhibitor such as EDTA, EGTA, etc., a serine protease inhibitor such as PMSF, trypsin inhibitor, chymotrypsin, etc., and/or a cysteine protease inhibitor such as iodoacetamide, E-64, etc., and a protease inhibitor cocktail commercially available from Sigma which contains such protease inhibitors premixed.

The phosphatase inhibitor is used for preventing the activated CDK, which is a protein itself, from changing its activity by hydrolysis of its phosphate group. Examples thereof include a serine/threonine phosphatase inhibitor such as sodium fluoride and a tyrosine phosphatase inhibitor such as sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ).

After the cells are solubilized, insoluble matters are

removed from cell lysate by centrifugation or by filtration using a filter. Subsequently, prior to separating the sample containing the activated CDK, it is preferable to measure the total amount of proteins in the cell lysate according to a method known to those skilled in the art. For example, the total amount of proteins may be measured using a DC protein kit using bovine IgG as a reference.

## (2) Separation of Sample Containing Activated CDK to Be Determined

The sample containing the activated CDK whose activity is to be determined is prepared from the thus obtained cell lysate. The sample containing the activated CDK can be prepared, for example, by an immunoprecipitation method.

According to the immunoprecipitation method, is used an anti-CDK antibody having a specificity to one of the CDKs 1 to 7 to be determined.

More particularly, the cell lysate containing a specific amount of protein is reacted with an anti-CDK antibody corresponding to the activated CDK to be determined and a suspension of sepharose beads (a beads content of 4 to 6 v/v%) coated with Protein A, Protein G or anti-rabbit IgG antibody as material for catching the anti-CDK antibody at 0 to 10 °C for one to two hours. Since these beads are insoluble, the complex of the anti-CDK antibody and the CDK bound to the beads become insoluble and precipitate.

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By immunoprecipitation, all CDK family (including the simple CDK, the activated CDK, the complex of the activated CDK and CDKI and the complex of the CDK and CDKI) in the liquid containing solubilized cells are caught. Accordingly, the activated CDK are contained together with the simple CDK, the complex of the activated CDK and CDKI and the complex of CDK and CDKI in the prepared sample. However, the inactivated CDK does not involve monothiophosphorylation of the substrate in the presence of ATP- $\gamma$  S. If the present invention is carried out on a sample containing the inactivated CDK for determining the activity of the activated CDK, the activity of the inactivated CDK is not detected and only the activity of the activated CDK is determined.

Subsequently, the precipitated beads to which the complex of the activated CDK and the anti-CDK antibody are bound are washed. A buffer solution for washing the beads contains magnesium chloride since the activated CDK needs to form a complex with magnesium in order that ATP- $\gamma$  S acts on the substrate and the activated CDK later. The buffer solution also contains, for example, dithiothreitol (DTT) as a stabilizer necessary for stabilizing the molecular structure of the substrate. Further the buffer solution may contain albumin, a trace of a surfactant and/or the like.

Thereafter, the activity of the activated CDK in the sample is determined. The method of the present invention

includes monothiophosphorylating the serine or threonine residue of the substrate in the presence of the activated CDK, labeling the resulting thiophosphorate group and measuring the label. In the present invention, the activated CDK bound to the CDK antibody caught by the beads may be used as an activated CDK.

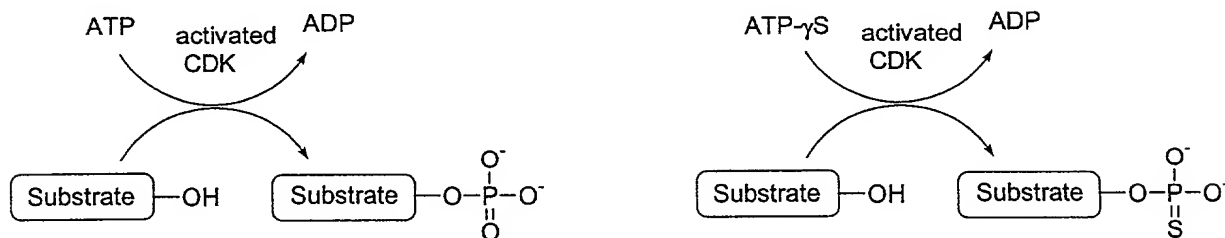
(i) In the presence of the activated CDK, a substrate, which is a substrate for the CDK, is reacted with adenosine 5'-O-(3-thiotriphosphate) (ATP- $\gamma$  S) to introduce a monothiophosphate group derived from ATP- $\gamma$  S into the serine group and threonine group of the substrate.

Usually, activated CDKs act to react ATP with the serine or threonine group of the substrate to introduce a monophosphate group derived from ATP. However, in the present invention, ATP- $\gamma$  S is used instead of ATP to introduce the monothiophosphate group instead of the monophosphate group in the serine or threonine group of the substrate.

For monothiophosphorylation, a liquid of pH 6.5 to 8.5, preferably 7.4, containing 0.1 to 1.0 mg/mL of the substrate is reacted with 10 to 100 equivalents of ATP- $\gamma$  S with respect to 1 equivalent of the substrate in the presence of the activated CDK at 25 to 40°C, preferably 37°C, for 5 minutes to 1 hour, preferably 10 minutes.

As discussed above, the sample contains not only the activated CDK but also the inactivated CDK. However, since

only the activated CDK catalyzes the thiophosphate group introduction reaction, the inactivated CDK does not participate in the method of the present invention.



5

As examples of the substrate, histone H1 and Rb (Retinoblastoma protein) may be mentioned for the activated CDK1 and CDK2 and for the activated CD4 and CDK6, respectively.

10

In the present invention, regarding substrates such as Rb which essentially contain the cysteine residue in their molecules, the residue is substituted by an amino acid residue such as alanine which does not contain thiol group. This is for avoiding measurement errors owing to the labeling of the thiol group of the cysteine residue essentially present in the substrate at the same time when sulfur atom of thiophosphate group of the substrate (into which thiophosphate group derived from  $\text{ATP-}\gamma\text{S}$  by the action of the activated CDK) is labeled with the labeling fluorophore or the labeling enzyme.

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Regarding a substrate which essentially contains the cysteine residue in its molecules, it may be possible to produce,

from the substrate, a substrate of which the cysteine residue is substituted by an amino acid residue such as alanine which does not contain the thiol residue, by PCR or by modifying a gene of the substrate by site mutagenesis and expressing the modified gene. Particularly, with regard to a substrate such as Rb which contains the cysteine residue, a recombinant vector is obtained by cloning with use of oligonucleotide primers Rb-1 (5' - ACA GGA TCC TTG CAG TAT GCT TCC - 3'), Rb-2 (5' - GCT GTT AGC TAC CAT CTG ATT TAT - 3'), Rb-3 (5' - ATG GTA GCT AAC AGC GAC CGT GTG - 3') and Rb-7 (5' - GCG AAT TCA ATC CAT GCT ATC ATT - 3'); the recombinant vector is expressed to obtain a recombinant DNA in which a nucleotide coding cysteine residue is substituted by a nucleotide coding alanine residue; and the recombinant DNA is expressed to produce a substrate in which the cysteine residue is substituted by the alanine residue.

(ii) Labeling of the thiophosphate group introduced in the substrate and measurement of the amount of the label

In order to label the substrate by coupling the labeling fluorophore or the labeling enzyme with sulfur atom of the introduced thiophosphate group, a liquid of pH 7.5 to 9.0, preferably 8.5, containing 0.1 to 1.0 mg/mL of the substrate into which the thiophosphate group is introduced is reacted with 10 to 100 equivalents of a labeling fluorophore or a labeling enzyme having a functional group which reacts with

the thiol group, with respect to 1 equivalent of the substrate for 10 minutes to 2 hours. This reaction is stopped by adding a free thiol, for example,  $\beta$  - ME ( $\beta$  -mercaptoethanol), DTT (dithiothreitol) or the like.

5           In the case where the substrate is labeled with the labeling fluorophore, the amount of fluorescence from the labeling fluorophore is measured. The measured amount of fluorescence is compared with a reference curve which has been produced beforehand from the amount of fluorescence  
10   measured about the known amount of the substrate, and thereby the amount of the labeled substrate is calculated. The amount of the labeled substrate is regarded as an activity value of the activated CDK contained in the sample.

          In the case where the substrate is labeled with the  
15   labeling enzyme, the labeling enzyme is reacted with a substance which generates an optically detectable substance by reaction with the labeling enzyme. The amount of the generated product is optically measured and the measured amount is compared with a reference curve which has been  
20   produced beforehand, and thereby the activity value of the activated CDK contained in the sample is calculated. Here the optically detectable substance means a substance whose existence can be detected by measuring fluorescence, absorbance and/or the like of the substance.

25           As examples of the labeling fluorophore capable of



binding to the sulfur atom of the thiophosphate group, may be mentioned fluorescein, coumarin, eosin, phenanthroline, pyrene, Rhodamine and the like, among which fluorescein is preferred. In order that the labeling fluorophores binds to the sulfur atom of the thiophosphate group, the labeling fluorophores have functional groups such as an alkyl halide, maleimide, aziridine site and the like which react with the thiol group for labeling the sulfur atom of the thiophosphate group.

As examples of the labeling fluorophore having a functional group which reacts with the thiol group, may be mentioned iodoacetyl-FITC (fluorescein isothiocyanate), 5-(bromomethyl)fluorescein, fluorescein-5-maleimide, 5-iodoacetamidfluorescein (5-IAF), 6-iodoacetamidfluorescein (6-IAF), 4-bromomethyl-7-methoxycoumarin, eosin-5-iodoacetamide, eosin-5-maleimide, eosin-5-iodoacetamide, N-(1,10-phenanthroline-5-yl)bromoacetamide, 1-pyrenebutylchloride, N-(1-pyreneethyl)iodoacetamide, N-(1-pyrenemethyl)iodoacetamide (PMIA amide), 1-pyrenemethyliodoacetate (PMIA ester), Rhodamine red C2 maleimide and the like, among which iodoacetyl-FITC is preferable.

Alternatively, the labeling fluorophore may be introduced to thiophosphate group by reacting the molecule with biotin which has a functional group reacting with sulfur

atom of thiophosphate group, for example, iodoacetylbiotin, and then reacting the molecule with a labeling fluorophore covalent-bound to avidin for taking advantage of the affinity of biotin to avidin.

5           The labeling enzyme may be introduced to sulfur atom of thiophosphate group by introducing iodoacetylbiotin to sulfur atom and then reacting the molecule with a labeling enzyme covalent-bound to avidin which has affinity to biotin. As such enzymes, may be mentioned  $\beta$  galactosidase, alkaline  
10   phosphatase, peroxidase and the like, among which peroxidase is preferred.

          The amount of the labeled substrate may be measured by measuring the amount of fluorescence from the labeling fluorophore or by allowing a substance which generates an  
15   optically detectable product by reaction with the labeling enzyme to act on the substrate labeled with the labeling enzyme and then optically measuring the generated product.

          More particularly, where the labeling fluorophore is used, the labeling fluorophore is excited by a specific  
20   wavelength and analyzed by a fluorescent image analyzer. The wavelength of applied light may vary depending upon the type of a labeling fluorophore used. For example, light of 488 nm wavelength is applied for excitation where the labeling fluorophore is fluorescein.

25           Where the labeling enzyme is used, a substrate which

will produce a fluorophore by reaction with the labeling enzyme is added to the substrate labeled with the labeling enzyme in order to produce the fluorophore by reaction with the labeling enzyme. The produced fluorophore is excited by light having  
5 a specific wavelength and the emitted fluorescence is detected. The substrate which produces a fluorophore by reaction with the labeling enzyme may be ECL-plus in the case where the labeling enzyme is peroxidase. The substrate may be selected as appropriate depending upon a labeling enzyme used.

10 The amount of the labeling fluorophore or the amount of the fluorophore produced by the reaction is measured and applied to the reference curve made beforehand in order that the activity of the activated CDK is calculated. The reaction liquid of the labeled substrate needs to be diluted to such a  
15 degree that the amount of fluorescence from the labeling fluorophore or the fluorophore produced by the reaction with the labeling enzyme falls within the range of the reference curve. For example, the reaction liquid may be diluted 100 to 500 fold. As diluents, may be used TBS (50 mM Tris-HCl of  
20 pH 7.5, 150 mM NaCl), water, an aqueous sodium chloride solution and the like. In the case of aqueous sodium chloride solution, the concentration of sodium chloride may preferably be in the range of 100 to 500 mM. In the case where the reaction liquid is diluted, the dilution is taken into account  
25 when the activity of the activated CDK is calculated. The

resulting activity of the activated CDK is the activity of the activated CDK in a specific amount of protein taken from the total protein of the prepared sample.

The reference curve is preferably produced beforehand using a known amount of the substrate to which thiol group is introduced. Also biotinylated actin may be used instead. Biotinylated actin is known to have the same behavior to reaction with the labeling fluorophore and the labeling enzyme as the substrate to which thiol groups has been introduced.

10 In this case, the activity of the activated CDK is required to be calculated from the amount of biotinylated actin.

The present invention also provides a method for diagnosing cancers such as stomach cancer, colon cancer, breast cancer, lung cancer, esophageal cancer, prostate cancer, hepatic cancer, kidney cancer, bladder cancer, skin cancer, uterine cancer, cerebral tumor, osteosarcoma and myeloma, from the results of the CDK activity determined by the determination method of the present invention.

## 20 Examples

Exemplary Method 1 (Determination of Activated CDK1 : using histone H1 as a substrate and utilizing peroxidase-labeling)

First Step :

In ice bath, HeLa cells (carcinoma cells of uterine cervix) were lysed in a lysis buffer containing 0.1 w/v% NP40

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(surfactant Nonidet P-40), 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate and 100  $\mu$  L/mL protease inhibitor cocktail (Sigma), in a proportion of  $1 \times 10^7$  cells/5 mL buffer, by 10 times repeated sucking and discharging with a 5-mL syringe provided with a 23G needle. A cell lysate was thus prepared. Insolubles were removed by centrifugation at 4  $^{\circ}$ C at 15,000 rpm for 5 minutes. The total amount of protein contained in the supernatant was measured by a DC protein kit (Bio-Rad) using bovine IgG as reference.

#### Second Step :

A sample was prepared by adding 10  $\mu$  g of the lysed protein in the total amount to 500  $\mu$  L of the lysis buffer in an Eppendorf tube of 1.5 mL volume. To the prepared sample, 10  $\mu$  L of polyclonal anti-CDK antibody (Santa Cruz Biotechnology) were added. A 1 : 1 (sepharose beads : lysis buffer) slurry of 60  $\mu$  L sepharose beads (finally 30  $\mu$  L of enclosed beads) coated with Protein A was added to the resulting sample, which was incubated at 4  $^{\circ}$ C for an hour with continuous rotation. The beads were taken out of the sample and washed with 1 mL of the lysis buffer twice. Then the beads were washed once with 1 mL of a kinase buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM magnesium chloride ( $\text{MgCl}_2$ ) and 1 mM DTT. The beads were suspended again in 15  $\mu$  L of the kinase buffer.

Third Step :

To the obtained suspension, 10  $\mu$  L of a histone H1 solution (0.1 mg/mL solution in 50 mM Tris-HCl, pH 7.4) were added. Then 10  $\mu$  L of an ATP- $\gamma$  S solution (10 mM aqueous solution) were added to the suspension. The resulting suspension was incubated at 37  $^{\circ}$ C for 10 minutes with continuous oscillation. The beads were precipitated by centrifugation at 10,000 rpm for 10 seconds and 30  $\mu$  L of supernatant were collected. To the supernatant, were added 25  $\mu$  L of a binding buffer containing 150 mM Tris-HCl and 5 mM EDTA of pH 9.2 since pH 8.5 is the optimum condition for binding reaction of iodoacetylbiotin with thiophosphoric acid. To the resulting supernatant, 20  $\mu$  L of a 40 mM PEO-iodoacetylbiotin (Pierce) solution (in a 20 mM phosphate buffer of pH 6.0) were added. The supernatant was incubated in a dark place at room temperature for 90 minutes. The reaction with iodoacetylbiotin was stopped by adding 7.5  $\mu$  L of  $\beta$ -ME ( $\beta$ -mercaptoethanol). The reaction liquid was diluted with TBS (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride).

Fourth Step :

The diluted reaction liquid, 50  $\mu$  L, was placed and absorbed onto a PVDF membrane using a slot blotter. The membrane was washed once with 50 mL of TBS-T (a TBS solution containing 0.05 w/v% Tween 20). In order to prevent the membrane to react with avidin-peroxidase, a hydrophobic

part of the membrane was blocked with BSA (bovine serum albumin) beforehand. More particularly, the membrane was blocked with 3 w/v% of BSA in TBS-T at room temperature for 30 minutes. The membrane was reacted with

5 avidin-peroxidase (Vector) (50,000-fold diluted with TBS-T) at room temperature for 10 minutes. The membrane was washed with 50 mL of TBS-T three times. Then the membrane was reacted with ECL-plus (Amersham) for 5 minutes. A solution of ECL-plus was prepared according to the manufacturer's

10 instructions. The reaction was stopped by washing the membrane with 200 mL of water. Bands of fluorescence were visualized by Molecular Imager (Bio-Rad) and quantified.

Exemplary Method 2 (Determination of Activated CDK1 : using

15 histone H1 as a substrate and utilizing FITC-labeling)

This method was carried out in the same manner as Exemplary Method 1 except that avidin-FITC was used in place of avidin-peroxidase and ECL-plus was not reacted with the membrane in the above fourth step.

Exemplary Method 3 (Determination of Activated CDK2 : using

20 histone H1 as a substrate and utilizing FITC-labeling)

First Step :

Tissue having a wet weight of 10 mg to 50 mg was put

25 in a Eppendorf tube (1.5 mL volume), to which 800  $\mu$  L of the

lysis buffer mentioned in the first step of Exemplary Method 1  
were added. The mixture was ground down with a pestle. A  
basic movement of the pestle turning right to left at 90° at a  
pressing force of 5 kg was repeated 10 times. The resulting  
5 crude liquid of solubilized cells was passed through a syringe  
(1 mL volume) fed with glass wool (about 0.1 g weight) and  
provided with a disk filter (Milipore) having a pore size of 0.45  
 $\mu$  m at the tip thereof. Thereby was prepared a liquid of  
solubilized cells from which insolubles and lipid were removed.  
10 The total amount of protein contained in the supernatant was  
measured by a DC protein kit (Bio-Rad) using bovine IgG as  
reference.

#### Second Step :

The Second Step was carried out in the same as in the  
15 second step of Exemplary Method 1.

#### Third Step :

To the suspension, 10  $\mu$  L of a histone H1 solution  
(0.1 mg/mL solution in 50 mM Tris-HCl, pH 7.4) were added.  
Then 10  $\mu$  L of an ATP- $\gamma$  S solution (10 mM aqueous solution)  
20 were added to the suspension. The resulting suspension was  
incubated at 37 °C for 90 minutes with continuous oscillation.  
The beads were precipitated by centrifugation at 1,000 rpm for  
10 seconds and 30  $\mu$  L of supernatant were collected. To 18  $\mu$   
L of the supernatant, were added 15  $\mu$  L of a binding buffer  
25 containing 150 mM Tris-HCl, pH 9.2 and 5 mM EDTA. To the



resulting supernatant, 10  $\mu$  L of a 5 mM iodoacetylfluorecein (Pierce) solution (in a 50 mM phosphate buffer of pH 6.0 and 50 % dimethylsulfoxide) were added. The mixture was incubated in a dark place at room temperature for 90 minutes.

- 5 The reaction of iodoacetamidofluorecein was stopped by adding 43  $\mu$  L of  $\beta$ -ME. The reaction liquid was diluted 5 fold to 10 fold with TBS (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride).

Fourth Step :

- 10 The diluted reaction liquid, 50  $\mu$  L, was placed and absorbed onto a PVDF membrane using a slot blotter. The membrane was washed with 50 mM TBS-T (a TBS solution containing 0.05 w/v% Tween 20) for 10 minutes three times with oscillation. Thereafter the membrane was washed with  
15 200 mL of water and dried. Bands of fluorescence were visualized by Molecular Imager (Bio-Rad) and quantified by an image analyzer.

- Exemplary Method 4 (Determination of Activated CDK2 : using  
20 histone H1 as a substrate and utilizing FITC-labeling)

First Step :

This step was carried out in a manner similar to the first step of Exemplary Method 1 using K562 cell line.

Second Step :

- 25 Samples were prepared in graded concentrations of 0,

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25, 50, 100 and 200  $\mu$ g/mL of the total amount of protein of solubilized K562 cells in 500 L of the lysis buffer and put in Eppendorf tubes. To each of the samples, 10  $\mu$  L of polyclonal anti-CDK antibody (200  $\mu$ g/mL, Santa Cruz Biotechnology) were added. To the resulting samples, a 1 : 1 (sepharose beads : lysis buffer) slurry of 40  $\mu$  L sepharose beads coated with Protein A was added. The samples were incubated at 4 °C for an hour with continuous rotation. The beads were taken out of the samples and washed with 1 mL of the lysis buffer twice. Then the beads were washed once with 100 mM Tris-HCl of pH 7.4 and 100 mM sodium chloride and further with 100 mM Tris-HCl of pH 7.4.

#### Third Step :

To the beads, 50  $\mu$  L of kinase buffer solution containing histone H1 (40 mM Tris-HCl, pH7.4, 18 mM magnesium chloride, 2 mM ATP- $\gamma$  S, 6  $\mu$ g/test histone H1) were added. The resulting suspension was incubated at 37 °C for 90 minutes with continuous oscillation. The beads were precipitated by centrifugation at 1,000 rpm for 10 seconds and 36  $\mu$  L of supernatant were collected. To 36  $\mu$  L of the supernatant, were added 30  $\mu$  L of a binding buffer containing 150 mM Tris-HCl and 2.5 mM EDTA of pH 9.2. Further, 20  $\mu$  L of a 35 mM PEO-iodoacetylbiotin (Pierce) solution (in a 50 mM phosphate buffer of pH 6.0) were added. The resulting mixture was incubated in a dark place at room temperature for

90 minutes. The reaction was stopped by adding an equivalent amount (86  $\mu$  L) of  $\beta$ -ME. The reaction liquid was diluted 5 fold to 10 fold with TBS (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride).

5 Fourth Step :

The diluted reaction liquid, 50  $\mu$  L, was placed and absorbed onto a PVDF membrane using a slot blotter. The obtained membrane was blocked with 1 w/v% BSA for 30 minutes and washed with TBS for 5 minutes. Reaction was  
10 conducted in a solution of avidin-FITC (Pierce) (500-fold diluted with TBS) at 37°C for 60 minutes. After the reaction, the membrane was washed with TBS three times and with water once, and dried. Bands of fluorescence were visualized by Molecular Imager (Bio-Rad) and measured.

15

Production Example 1 (Production of a recombinant vector coding for a Rb (retinoblastoma protein) whose cysteine residue is substituted by alanine residue and a protein produced by expression of the vector.

20 (1) Construction of Expression Vector

First a cDNA coding human Rb was cloned from a cDNA library (Stratagene) of human placenta.

For constructing a plasmid for expressing a C-terminal (from Leu 769 to Lys 928) of human Rb whose Cys  
25 853 was uniquely modified to Ala, a two-stage PCR was carried

out using oligonucleotide primer with pJ3 $\Omega$  vector containing the full length of cDNA of human Rb.

#### 1. First PCR

First, for amplifying a region of human Rb protein  
5 corresponding to Leu769 to Asp921 and substituting Cys 853  
by Ala at the same time, the two-stage PCR was carried out  
using four species of primers. The primers used were primer  
Rb-1 (5' - ACA GGA TCC TTG CAG TAT GCT TCC - 3', into  
which a BamHI site (as underlined) was introduced) and Rb-7  
10 (5' - GCG AAT TCA ATC CAT GCT ATC ATT - 3', into which a  
EcoRI site (as underlined) was introduced), which were primers  
at both ends, a primer Rb-2 whose 853 position was changed  
into Ala codon (AGC) (5' - GCT GTT AGC TAC CAT CTG ATT  
TAT - 3', the point modified codon is shown as underlined) and  
15 its complementary primer Rb-3 (5' - ATG GTA GCT AAC AGC  
GAC CGT GTG - 3'). The PCR was carried out with a primer  
set of Rb-1/Rb-2 and a primer set of Rb-3/Rb-7 using the total  
length of cDNA of human Rb as a template under the following  
reaction conditions, to obtain PCR fragments 1 and 2, which  
20 were complementary in regions corresponding to the primer  
Rb-2 and the primer Rb-3.

Composition of Reaction Liquid (for fragment 1 corresponding  
to nucleotide 2305 to 2565)

PJ3 $\Omega$ -Rb	250 ng
25 Taq DNA polymerase (TaKaRa Ex Taq, Takara Shuzo)	0.03U

Buffer for TaKaRa Ex Taq (Takara Shuzo)

MgCl<sub>2</sub> (Takara Shuzo) 2 mM

dNTPs (Takara Shuzo) 250 μM

Primer Rb-1 1 μM

5 Primer Rb-2 1 μM

Total 50 μL

Composition of Reaction Liquid (for fragment 2 corresponding to nucleotide 2551 to 2763)

PJ3 Ω-Rb 250 ng

10 Taq DNA polymerase (TaKaRa Ex Taq, Takara Shuzo) 0.03U

Buffer for TaKaRa Ex Taq (Takara Shuzo)

MgCl<sub>2</sub> (Takara Shuzo) 2 mM

dNTPs (Takara Shuzo) 250 μM

Primer Rb-3 1 μM

15 Primer Rb-7 1 μM

Total 50 μL

Reaction Temperature

At 95°C for 5 minutes

At 94°C for 30 seconds, at 55°C for 1 minute, at 72°C for 1

20 minute (15 cycles)

At 72°C for 2 minutes

2. Treatment of Overhang

A-3' overhangs of the PCR products were treated with Klenow under the following conditions.

### Composition of Reaction Liquids

PCR fragment 1 and 2

Klenow fragment (Takara Shuzo) 0.07U

Buffer for Klenow fragment (Takara Shuzo)

5 dNTPs (Takara Shuzo) 250  $\mu$  M

### Reaction Temperature

At 37°C for 1 hour

### 3. Second PCR

10 Thereafter, using a mixture of the PCR products as a template, PCR was carried out with a primer set of Rb-1/Rb-7 at both ends under the following reaction conditions, to amplify a DNA fragment of 470 bp corresponding to Leu769 to Asp921 in which Cys 853 was substituted by Ala.

### Composition of Reaction Liquid

15 Klenow-treated PCR fragment 1 and 2

Taq DNA polymerase (TaKaRa Ex Taq, Takara Shuzo) 0.03U

Buffer for TaKaRa Ex Taq (Takara Shuzo)

MgCl<sub>2</sub> (Takara Shuzo) 2 mM

dNTPs (Takara Shuzo) 250  $\mu$  M

20 Primer Rb-1 1  $\mu$  M

Primer Rb-7 1  $\mu$  M

Total 50  $\mu$  L

### Reaction Temperature

At 95°C for 5 minutes

25 At 94°C for 30 seconds, at 55°C for 1 minute, at 72°C for 1

minute (15 cycles)

At 72°C for 2 minutes

#### 4. Cloning into pMelBacA

In order to express a Rb protein having a secretion  
5 signal added at the N terminal, after the DNA fragment of 470  
bp amplified in the previous step 3 was digested with BamHI  
and EcoRI, the digested fragment was inserted at the BamHI  
site and at the EcoRI site of pMelBacA (Invitrogen). The  
obtained plasmid was referred to as pMelBacA-Rb.

#### 10 5. PCR

Using the obtained plasmid as a template, PCR was  
carried out using primer Rb-9 (5' - GCG AAT TCA TGA AAT TCT  
TAG TCA - 3', into which the EcoRI site was introduced as  
underlined) and primer Rb-5 (5' - GTT CTC GAG TCA ATC CAT  
15 GCT ATC ATT - 3', into which the XhoI site was introduced as  
underlined) under the following conditions, to amplify the DNA  
fragment of 540 bp to which the secretion signal was added.

#### Composition of Reaction Liquid

	pMelBacA-Rb	250 ng
20	Taq DNA polymerase (TaKaRa Ex Taq, Takara Shuzo)	0.03U
	Buffer for TaKaRa Ex Taq (Takara Shuzo)	
	MgCl <sub>2</sub> (Takara Shuzo)	2 mM
	dNTPs (Takara Shuzo)	250 $\mu$ M
	Primer Rb-9	1 $\mu$ M
25	Primer Rb-5	1 $\mu$ M

Total 50  $\mu$  L

Reaction Temperature

At 94°C for 5 minutes

At 94°C for 30 seconds, at 55°C for 1 minute, at 72°C for 1  
5 minute (25 cycles)

At 72°C for 2 minutes

6. Cloning into pFastBac1

The DNA fragment of 540 bp containing the secretion  
signal amplified in the previous step 5 was digested with EcoRI  
10 and XhoI, and then was inserted at the EcoRI site and at the  
XhoI site of pFastBac1 (Lifetech).

(2) Isolation of Recombinant Virus by Bac-To-Bac Baculovirus  
Expression System (Lifetech)

The expression plasmid obtained in the previous step  
15 6 was used for isolating a recombinant virus, that is, according  
to the manufacturer's instructed protocol.

(3) Expression

The liquid of the recombinant virus prepared in the  
previous step (2) was infected to insect cells (High Five Cell,  
20 Invitroge) under the condition of MOI=10 to express a region  
corresponding to Leu 769 to Asp 921 containing Cys 853  
substituted by Ala. The secretion of the expressed protein  
into a medium (CELL405, JRH Biosciences) was confirmed by  
Western blotting using an anti-human Rb polyclonal antibody  
25 (Rb(C-15), Santa Cruz), and the medium was harvested five



days after the infection.

#### (4) Purification of Expressed Protein

The medium containing the Rb recombinant protein obtained in the previous step (3) was exchanged with 50 mM MES buffer (pH 6.0) by PD-10 column (Pharmacia), and then the protein is eluted by CM-5pW column (Tosoh) using a linear gradient of 0 to 1 M NaCl. The elution of the Rb recombinant protein at about 0.3 M NaCl was confirmed by Western blotting using an anti-human Rb polyclonal antibody (Rb(C-15), Santa Cruz).

The obtained protein was used in place of histone H1 as a substrate in the third step of Exemplary Method 1 for determination of CDK4 or CDK6.

Exemplary Method 5 (Determination of Activated CDK4 : utilizing the recombinant human Rb produced in Production Example 1 as a substrate and FITC-labeling  
First Step :

This step was carried out in the same manner as in the first step of Exemplary Method 3.

Second Step :

Samples were prepared by placing the solubilized K562 cells and the lysis buffer into Eppendorf tubes of 1.5 mL volume so that 0, 50, 100, 125 and 250  $\mu$ g of the total amount of protein were in 500  $\mu$ L of the lysis buffer. To each of the

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samples, 10  $\mu$  L of a polyclonal anti-CDK 4 antibody (200  $\mu$  g/mL Santa Cruz Biotechnology) were added. A 1 : 1 (sepharose beads : lysis buffer) slurry of 40  $\mu$  L sepharose beads coated with Protein A was added to the resulting  
5 samples, which were incubated at 4  $^{\circ}$ C for an hour with continuous rotation. The beads were taken out of the samples and washed with 1 mL of the lysis buffer twice. Then the beads were washed once with 100 mM Tris-HCl of pH 7.4 and 100 mM sodium chloride and further washed once with 100  
10 mM Tris-HCl of pH 7.4.

Third Step :

To the obtained beads, 50  $\mu$  L of phosphorylation solution containing the recombinant human Rb protein produced in Production Example 1 (40 mM Tris-HCl, pH 7.4, 15 200 mM magnesium chloride, 3.3 mM ATP- $\gamma$  S, 20  $\mu$  L recombinant human Rb protein solution : 50 mM MES buffer, pH 6.0) were added. The resulting suspension was incubated at 37  $^{\circ}$ C for 90 minutes with continuous oscillation. The beads were precipitated by centrifugation at 1,000 rpm for 10  
20 seconds and 36  $\mu$  L of supernatant were collected. To 36  $\mu$  L of the supernatant, were added 30  $\mu$  L of a binding buffer containing 150 mM Tris-HCl and 2.5 mM EDTA of pH 9.2. To the resulting supernatant, 20  $\mu$  L of a 50 mM PEO-iodoacetylbiotin (Pierce) solution (in a 50 mM phosphate  
25 buffer, pH 6.0). The supernatant was incubated in a dark

place at room temperature for 90 minutes. Thereafter, the supernatant was treated with an equivalent amount (86  $\mu$  L) of an SDS-sample loading buffer (0.125 M tris-HCl, pH6.8, 4% SDS, 10 %  $\beta$ -ME, 25 % glycerine, bromophenol blue) at 100°C for 5 minutes.

#### Fourth Step :

The samples prepared in the third step was subjected to SDS-PAGE under the condition of 20  $\mu$  L/lane (pre-cast gel, 4-20 % gradient, 10 mm  $\times$  10 mm, Daiichi Kagaku Yakuhin). The conditions of the SDS-PAGE were in compliance with the instruction protocol of Daiichi Kagaku Yakuhin (60 mA, 40 minutes). After the SDS-PAGE, the protein extended in the gel was electrically transferred to a PVDF membrane (10V, 30 minutes, Western blotting). The obtained membrane was blocked with 4 w/v % BSA for 30 minutes and washed with TBS-T for 5 minutes. Subsequently, the membrane was reacted in a solution of avidin FITC (Pierce) (diluted 1,000 fold with TBS-T) at 37°C for 30 minutes. After reaction, the membrane was washed with TBT-T twice and with water once, and dried. Bands of fluorescence were visualized by Molecular Imager (Bio-Rad) and measured.

#### Determination of Activity of Activated CDK1

1. Production of reference curve using biotinylated actin (BA) as reference substance

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Samples of BA are prepared in graded concentrations from 0 to 1000 ng/mL and each of the samples, 50 mL, is put into a slot. The sample in the slot is treated as in the fourth step of Exemplary Method 1. Since BA is labeled with

5 peroxidase, BA produces a fluorophore from ECL-plus (fluorescent substrate) in the fourth step. The amount of fluorescence is measured for the fluorophores generated in the samples containing the graded concentrations of BA and is indicated by count (CNT) values. The obtained data are

10 plotted with the BA concentration in abscissa and the CNT value in ordinate, and a reference curve is produced under the reaction conditions of Exemplary Method 1. Fig. 1 shows the obtained reference curve.

In the same manner, the samples in the slots are

15 treated as in the fourth step of Exemplary Method 1, and a reference curve is produced in the above-described manner under the reaction conditions of Exemplary Method 2. Fig. 2 shows the obtained reference curve.

## 2. Calculation of Activity of Activated CDK1

20 With regard to Exemplary Methods 1 and 2, blank samples are prepared which are treated according to Exemplary Method 1 except that the anti-CDK antibody is not added. The amount of fluorescence from the blank samples is measured. The obtained CNT values are converted to BA

25 concentrations using the reference curves produced for

Exemplary Methods A and B as described above. The activity of the activated CDK is calculated by substituting the obtained BA concentrations into the following formula:

Formula 1 : {(the converted BA concentration of a sample) - (the converted BA concentration of the blank sample)} × (the dilution ratio of the sample) = (the activity of the activated CDK1)

If the produced reference curve is not a sigmoid curve but a linear line (e.g., Exemplary Method 2), the activity of the activated CDK may be calculated by calculating (the CNT value of a sample) - (the CNT value of the blank sample), converting the obtained remainder to BA concentration using the reference curve and substituting the converted BA concentration into the following formula:

Formula 2 : (the converted BA concentration of the remainder of the CNT value of the sample minus the CNT value of the blank sample) × (the dilution ratio of the sample) = (the activity of the activated CDK)

Example 1 (Measurement of activity of activated CDK1 of sample treated according to Exemplary Method 1)

The amount of fluorescent of 450-fold diluted samples prepared by Exemplary Method 1 using the anti-CDK1 antibody was measured as described above. Sample 1 was prepared using HeLa cells in a growth phase and Sample 2 was prepared using HeLa cells in a stationary phase. Since the

reference curve was a sigmoid curve as shown in Fig. 1, the activity of the activated CDK1 was calculated using Formula 1, that is, by producing blank samples as described above, measuring the amount of fluorescence of the blank samples and substituting the CNT values of the blank samples and the samples into Formula 1. The results are shown in Table 2.

Table 2

	CNT value	Activity of activated CDK1 (ng/slot)
Sample 1	147.6	6921.4
Blank of Sample 1	68.7	-
Sample 2	133.3	5121.85
Blank of Sample 2	69.1	-

Example 2 (Determination of activity of activated CDK1 of sample treated according to Exemplary Method 2)

The amount of fluorescent of 100-fold diluted samples prepared by Exemplary Method 2 using the anti-CDK1 antibody was measured as described above. Since the reference curve was linear as shown in Fig. 2, it was found that the activity of the activated CDK1 was able to be calculated using any one of Formula 1 and Formula 2.

#### Determination of Activity of Activated CDK2

Example 3 (Determination of activity of activated CDK2 of samples treated by Exemplary Method 3)

Sample 3 was prepared using the anti-CDK2 antibody according to Exemplary Method 3. As controls, Sample 4 was prepared according to Exemplary Method 3 without using the anti-CDK2 antibody, and Sample 5 was prepared according to Exemplary Method 3 except that a non-specific IgG antibody was used instead of the anti-CDK2 antibody in the second step. The fluorescence bands of Samples 3 to 5 are shown in Fig. 3. The amount of fluorescence of the bands were numerically represented by Molecular Imager (Bio-Rad). The obtained amount of fluorescence is shown in Table 3.

Table 3

	CNT value
Sample 3	363
Sample 4	198
Sample 5	192

The above results show that a non-specific reaction was not observed in the case where the non-specific IgG antibody was added instead of the anti-CDK2 antibody (Sample 5) and therefore that the activity of the activated CDK2 was able to be determined by the method of the present invention.

Example 4 (Determination of activity of activated CDK2 of sample treated by Exemplary Method 4)

1. Production of reference curve using K562 cell line (premyelocytic leukemia) as reference substance

The amount of fluorescence was measured about samples having graded concentrations of 0 to 200  $\mu$ g/mL produced according to Exemplary Method 4 and represented by CNT values. The obtained data were plotted with the concentration of K562 cell line in abscissa and the CNT value in ordinate to produce a reference curve. The obtained reference curve is shown in Fig. 4.

## 2. Calculation of Activity of Activated CDK2

A specimen was prepared according to Exemplary Method 4 except that an unknown sample was used instead of the sample of solubilized K562 cells. The amount of fluorescence of the specimen was measured, and the obtained CNT value was converted to a CDK2 activity of the solubilized K562 cells using the above-produced reference curve to calculate the activity.

## Determination of Activity of Activated CDK4

Example 5 (Determination of activity of activated CDK4 of samples treated by Exemplary Method 5)

Sample 6 was prepared according to Exemplary Method 5 using the anti-CDK4 antibody. As a control, Sample 7 was prepared according to Exemplary Method 5 except that the anti-CDK4 antibody was not used. The bands of fluorescence of Samples 6 and 7 are shown in Fig. 5.



### Example to Prove the Specificity of Activated CDK2

#### Measurements to Activity of CDK2 using CDK-specific Inhibitor

##### Example 6 (In the case of activated CDK2)

###### First Step :

- 5            This step was carried out in the same manner as in the first step of the Exemplary Method 1 using K562 cell line (premyelocytic leukemia).

###### Second Step :

- 10           A sample was prepared by placing the solubilized K562 cells and the lysis buffer into an Eppendorf tube of 1.5 mL volume so that 250  $\mu$ g of the total amount of protein of the solubilized K562 cells were in 500  $\mu$ L of the lysis buffer. To the sample, 10  $\mu$ L of a polyclonal anti-CDK2 antibody (200  $\mu$ g/mL, Santa Cruz Biotechnology) were added. A 1 : 1
- 15           (sepharose beads : lysis buffer) slurry of 40  $\mu$ L sepharose beads coated with Protein A was added to the resulting sample, which was incubated at 4 °C for an hour with continuous rotation. The beads were taken out of the sample and washed with 1 mL of the lysis buffer twice. Then the beads were
- 20           washed once with 100 mM Tris-HCl of pH 7.4 and 100 mM sodium chloride and further washed once with 100 mM Tris-HCl of pH 7.4.

###### Third Step :

- 25           As CDK inhibitors, were used Butyrolactone I (Calbiochem) which were inhibitors to CDK1 and CDK2 and

Staurosporine (Calbiochem) which had a broad inhibition spectrum including inhibition to CDK2. Phosphorylation solutions (40 mM Tris-HCl, pH 7.4, 18 mM magnesium chloride, 2 mM ATP- $\gamma$  S, 6  $\mu$ g/test histone H1), 50  $\mu$ L, containing

5 histone H1 and the CDK inhibitors (final concentrations of 0, 1, 10, 30, 100  $\mu$ M of Butyrolactone, or final concentrations of 0, 0.3, 1, 10, 30  $\mu$ M of Staurosporine) were added to the beads. The resulting suspensions were incubated at 37°C for 90 minutes with continuous oscillation. The beads were

10 precipitated by centrifugation at 1,000 rpm for 10 seconds, and 36  $\mu$ L of supernatant was collected. To 36  $\mu$ L of the supernatant, 30  $\mu$ L of a binding buffer containing 150 mM Tris-HCl and 5 mM of EDTA of pH 9.2 was added. Further, 20

15  $\mu$ L of 50 mM PEO-iodoacetylbiotin (Pierce) solution (50 mM phosphate buffer, pH 6.0) was added and the resulting mixture was incubated in a dark place at room temperature for 90 minutes. Thereafter, the mixture was treated with an equivalent amount (86  $\mu$ L) of an SDS-sample loading buffer (0.125 M tris-HCl, pH6.8, 4% SDS, 10 %  $\beta$ -ME, 25 % glycerine,

20 bromophenol blue) at 100°C for 5 minutes.

#### Fourth Step:

The samples prepared in the third step was subjected to SDS-PAGE under the condition of 20  $\mu$ L/lane (pre-cast gel, 4-20 % gradient, 10 mm  $\times$  10 mm, Daiichi Kagaku Yakuhin).

25 The conditions of the SDS-PAGE were in compliance with the

instructions of Daiichi Kagaku Yakuhin (60 mA, 40 minutes).  
After the SDS-PAGE, the protein extended in the gel was  
electrically transferred to a PVDF membrane (10V, 30 minutes,  
Western blotting). The obtained membrane was blocked with  
5 a 4 w/v % BSA for 30 minutes and washed with TBS-T for 5  
minutes. Subsequently, the membrane was reacted in a  
solution of avidin FITC (Pierce) (diluted 1,000 fold with TBS-T)  
at 37°C for 30 minutes. After reaction, the membrane was  
washed with TBS-T twice and with water once, and dried.  
10 Bands of fluorescence were visualized by Molecular Imager  
(Bio-Rad) and measured.

The visualized bands of fluorescence were numerically  
and graphically represented by an image analyzer. The  
results are shown in Fig. 6, wherein none represents the result  
15 of the above step without addition of the CDK inhibitors,  
DMSO represents the result of the above step with addition  
only of a solvent (dimethylsulfoxide) for the CDK inhibitors and  
IP (-) represents the result of the above step without addition  
of the CDK2 antibody. As shown in Fig. 6, the activity of  
20 CDK2 was inhibited dependently upon the amounts of the  
inhibitors. The results shows that the determined activity  
was specific to CDK2.

As shown above, the method of the present invention  
25 can measure the activity of cell cycle controlling factors

accurately without using radioisotopes.

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